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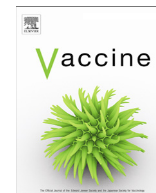

Peroxiredoxin 1 protein interacts with influenza virus ribonucleoproteins and is required for efficient virus replication

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ABSTRACT

Cellular proteins that support influenza virus infection represent potential therapeutic targets. Cytoplasmic egress intermediates of influenza A/WSN/33 were isolated and shown to be associated with the cellular enzymes peroxiredoxins-1 (Prdx-1) during glycerol gradient fractionation. Prdx-1 also co-localizes with influenza NP at the cell periphery late in infection. Knock-down or knockout of Prdx-1 expression inhibit influenza A replication. Inhibition of replication is not correlated with defects in initiation of infection or mRNA expression, but is correlated with inhibition of accumulation of viral proteins and vRNAs.

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1. Introduction

Influenza viruses are a major cause of morbidity and mortality. Unusually severe worldwide pandemics have occurred several times in the last hundred years and the possibility of another makes development and improvement of effective preventive and therapeutic strategies a critical public health concern. Currently licensed therapies for influenza infection target functionally conserved domains of viral proteins. Consequently, their use has selected for resistant viral variants that now circulate at high levels in the viruses that cause annual outbreaks [1–3]. Like all viruses, influenza subverts the functions of many viral proteins in order to complete a productive infection. Inhibition of cellular functions that are necessary for viral replication could presumably circumvent the problem of resistance selection, since the therapeutic target is not in the viral genome. The ideal cellular function for therapy could be inhibited transiently without serious toxic effects on the host.

Influenza viruses are unusual among RNA viruses in that they transcribe and replicate their genomes in the cell nucleus, but assemble viral particles at the plasma membrane of the infected cell. As such, movement of progeny viral genome-containing ribonucleoproteins (vRNPs) to the site of final assembly proceeds in two stages. In the first stage, progeny vRNPs must egress from the nucleus. This is accomplished by recruitment of viral genomes to the cellular CRM1/exportin1-dependent nuclear export pathway [4]. The current model for this process has the M1 C-terminal domain interacting with vRNPs, and the C-terminal domain of NEP interacting with the N-terminal domain of M1. NEP, in turn, can interact with CRM1 via a NES in its N-terminal domain creating a "daisy chain" connection between vRNPs and the CRM1 nuclear export apparatus [5,6]. Recently, a second CRM1-Dependent Nuclear Export Signal, located at the predicted N_2 helix of the N-terminal domain, was identified and its involvement in the nuclear export of vRNPs was demonstrated [7]. After egress from the nucleus, M1 protein apparently retains its association with the cytoplasmic vRNP since it has been shown to inhibit re-import of the vRNP [8,9]. There is evidence for cellular interactions at a later stage of nuclear egress. The drug leptomycin B inhibits nuclear export on the CRM1 pathway and consequently inhibits transport of NP complexes out of the nucleus [10–12]. Interestingly, in influenza virus-infected cells treated with Leptomycin B, NP complexes accumulate at the nuclear periphery and co-localize with the nuclear lamins suggesting that they may interact with components of the nuclear lamina as part of the egress pathway. There is no evidence in the literature for nuclear lamina participation in nuclear export, but viral capsids present a special problem in export, since they are larger than most cargo routed for export and may require a special pathway for transport from their site of synthesis to the nuclear envelope.

Although there is as yet little evidence for involvement of cellular factors in pre-budding cytoplasmic stages of egress, it seems highly likely that cellular factors play critical roles. Like most virus capsid assemblies, influenza NP complexes are too large to diffuse rapidly and yet move relatively rapidly from the nucleus to the

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plasma membrane. It is likely that the vRNPs may interact at least with host cytoplasmic transport machinery to accomplish this. NP, when expressed alone, can associate with actin, but it is as yet unclear whether vRNPs do so in infection. Some of these cellular components become part of the mature virion and may contribute to the infectivity of the virion. Here, we have purified cytoplasmic vRNPs from influenza virus-infected cells for proteomic analysis and identified a co-purifying cellular factor, peroxiredoxin-1 (Prdx-1).

Prdx-1 (also known as Prx-1, Prx1 and PAG) is one of a family of proteins first characterized for their ability to reduce hydrogen peroxide to water. Two members of this family, Prdx-1 and Prdx-2, are abundant cytoplasmic and nucleoplasmic enzymes with high sequence similarity [13,14]. Other peroxiredoxins may be localized to the mitochondria or secreted to the extracellular environment. Peroxiredoxins serve several functions in the cell. First, reduction of peroxide prevents over-accumulation of destructive reactive oxygen species (ROS) in the cell as a whole and peroxiredoxins, along with catalase and glutathione peroxidases helps maintain low concentration of ROS in the cell. Three other functions of peroxiredoxins have been identified that allow Prdx regulation of the activities of individual proteins and protein complexes. (i) Prdx enzymes are thought to control local H_2O_2 concentrations to modulate a variety of signaling pathways [15,16]; (ii) Prdx-1 and Prdx-2 can form decamers and other, higher order multimers that have chaperone activity [17,18]. (iii) Prdx-1 has been shown to interact directly with, and regulate the activity of cellular signaling molecules including the protein kinases c-Abl and JNK, the protein tyrosine phosphatase and tumor suppressor PTEN, the transcription activator c-myc, and androgen receptor [19–22]. As such, Prdx-1 may represent an attractive target for anti-influenza therapy.

2. Material and methods

2.1. Cells and viruses

Madin-Darby canine kidney (MDCK) and 293T cells were cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum. Human lung carcinoma A549 cells were cultured in Eagle minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Prdx1 −/− (Prdx1 knockout) and Prdx-1 +/+ (wild type) mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum. Influenza A/WSN/33 was the kind gift of Kevin Legge.

2.2. Viral growth and titer determination

Influenza A/WSN/33 virus was cultured in 10-day-old specific – pathogen-free embryonated chicken eggs (Charles River) for 2–3 days at 37 °C. Infected egg allantoic fluid was collected and cleared by centrifugation at 1000 rpm for 10 min. The supernatant was collected and stored at −80 °C.

Virus titers were determined by plaque assay. Briefly, confluent monolayers of MDCK cells were infected with 10-fold serial dilution of virus suspension for 1 h at 37 °C. Virus inoculum was then removed and replaced with warmed Dulbecco's' modified Eagle's media containing 1% low-temperature agarose. The medium was allowed to solidify at RT and incubate for 3 days at 37 °C to promote plaque development. Cells were fixed with 25% formaldehyde, the solidified agarose was removed and cells were stained with a crystal violet solution. Plaques were counted and the virus titer was expressed as PFU/ml.

2.3. Isolation and characterization of vRNPs fraction

vRNP complexes were isolated from MDCK cells infected with A/WSN/33 virus at a multiplicity of 1 by a modification of previously described procedures for glycerol gradient fractionation of influenza vRNPs [23–25]. Uninfected and infected MDCK cells (typically 1×10^8) were infected with one PFU/cell of virus for 18 h. Cells were scraped into phosphate-buffered saline (PBS) and pelleted at low speed. Cells were disrupted by swelling on ice for 10 min in low salt buffer (10 mM NaCl, 0.2 mM EDTA, 20 mM Tris-HCl, pH 8.0) with protease inhibitor cocktail followed by homogenization with ten strokes of a Dounce homogenizer. Sucrose was immediately added to 250 mM to stabilize nuclei, and then the homogenate was centrifuged for 10 min at 10,000 rpm, to pellet nuclei and other large organelles. The supernatant was adjusted to 0.1% Triton X-100 to disrupt membranes and then centrifuged again for 5 h at 37,000 rpm in a SW40 rotor to pellet vRNPs and other large macromolecular complexes. The pellet was resuspended in a small amount of the same low-salt, detergent-containing buffer, layered onto a 15–30% glycerol gradient and centrifuged at 37,000 rpm for 1 h in an SW40 rotor. Fractions were collected from the top of the gradient. Proteins were precipitated by addition of the carrier protein lysozyme to 0.1 mg/ml and trichloroacetic acid (TCA) to a final concentration of 20%. After 30 min on ice the TCA precipitate was collected by centrifugation at 10,000 rpm in a microfuge for 5 min. The pellet was washed three times with cold acetone, dried and resuspended in SDS-PAGE sample buffer.

2.4. Peptide mass fingerprinting

Protein from glycerol gradient fractions were separated by SDS-PAGE and protein bands of interest were excised, reduced, alkylated and digested with trypsin. Peptide mass profiles were generated by MALDI-MS and analyzed using MASCOT (www.matrixscience.com).

2.5. Plasmid and cell lines

Hush 29mer shRNA constructs against Prdx-1 mRNA in pGEF-V-RS vector (Catalog number TG302287) and control shRNA constructs were purchased from OriGene. In order to construct stable cell lines expressing Hush shRNAs against Prdx-1, 293T cells were transfected with individual Hush shRNA constructs and then selected with 1 ug/mL puromycin for one week, and individual clones were isolated by limiting dilution. The shRNA vector plasmid also expresses EGFP. Green fluorescent cell clones were further screened by immunoblotting of total protein for Prdx-1.

2.6. Indirect immunofluorescence

Indirect immunofluorescence (IF) was performed for detection of influenza A virus NP and Prdx-1. Cells were fixed with 4% formaldehyde for 20 min, washed with phosphate-buffered saline (PBS) then permeabilized and blocked in the same step with IF buffer (0.5% Triton X, 0.5% Sodium Deoxycholate, 1% BSA, 0.05% Sodium Azide in PBS) for 1 h at RT. Primary antibodies were diluted in IF buffer as follows: mouse monoclonal anti-influenza A, nucleoprotein, clones A1 and A3 blend, 1:500 (Milipore); rabbit polyclonal anti-Prdx-1, ab59538-100 (Abcam), 1:400. Secondary antibodies were also diluted in IF buffer as follows: Alexa Flour 488 goat anti-mouse IgG (Invitrogen) diluted 1:500 was used to detect influenza A nucleoprotein, Alexa Flour 594 donkey anti-rabbit IgG (Invitrogen) diluted 1:500 was used to detect Prdx-1. Slo-fade II (Invitrogen) was used to mount cover slips on glass slides. All confocal microscopy work was done with a Zeiss 510 microscope.

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